14. (New) The antigen presenting vesicle according to Claim 13, wherein said vesicle does not contain a transferrin receptor.

REMARKS

The Present Invention

The claimed invention is directed to antigen presenting vesicles free from their natural surroundings. The invention is also directed to methods of obtaining vesicles and of using them to stimulate T cells.

The Pending Claims

Prior to entry of the above amendments, claims 2-4, 6 and 9-13 are pending. Claims 2-4, 6 and 13 are directed to an antigen presenting vesicle. Claims 9, 11-12 are directed to a method for obtaining antigen presenting vesicles. Claim 10 is directed to a method for stimulating a T cell.

The Office Action

Claims 9-12 are withdrawn from further consideration as being drawn to a nonelected invention.

The substitute specification must be accompanied by a statement stating that it contains no new matter.

A marked-up copy of the substitute specification is required.

Claims 2-4, 6 and 13 are rejected under 35 USC §112, first paragraph, enablement requirement.

Claims 2-4, 6 and 13 are rejected under 35 USC §112, first paragraph, written description requirement.

Claim 6 is rejected under 35 USC §112, second paragraph.

Claims 2-4, 6 and 13 are rejected under 35 USC §102(b), as being anticipated by Amigorena (*Nature* (1994) 369:113-120).

Amendments

Applicants have canceled Claims 2 and 10, amended Claims 9 and 11-13, and added new Claim 14.

Claims 9, 11 and 12 were amended to become product by process claims. These claims now therefore fall within the elected subject matter for this application. Support is found in originally filed Claims 9, 11 and 12, and on page 2, line 25 through page 3, line 7; page 8, line 23 through page 9, line 8; and page 11, line 36 through page 12, line 14 of the original specification.

Newly added Claim 14 recites an antigen presenting vesicle that does not contain a transferrin receptor. Support is found on page 4, lines 15-22.

In accordance with the Examiner's suggestion, Claim 13 was amended to recite "an antigen presenting cell". Support is found on page 5, line 35 through page 6, line 1 of the originally filed specification.

Applicants have made the indicated amendments to place this application in form for allowance or in better form for appeal. The amendments were not made earlier due to oversight or because they directly address the comments of the Examiner made in the most recent Official Action (paper 16). No new matter has been added by any of these amendments and the Examiner is respectfully requested to enter them.

Response

The Examiner's specific objections and rejections are reiterated below as small indented bold print, followed by Applicant's response in normal print.

Objections

Applicant's amendment, mailed 10-27-00 (Paper No. 15), is acknowledged. Claims 2-4, 6 and 9-13 are pending, Claims 9-12 are withdrawn from further consideration by the examiner, 37 C.F.R. § 1.142(b) as being drawn to a nonelected invention. Claims 2-4, 6 and 13 are presently under consideration. A complete reply to the final rejection must include cancellation of non-elected claims or other appropriate action (37 CFR 1.144) See MPEP §821.01

Applicants have canceled Claims 2 and 10, and amended Claims 9, 11 and 12 to be product by process claims directed to an antigen presenting vesicle.

A substitute specification is required because the numerous entries directed by Applicant's amendment, mailed 10-27-00 (Paper No. 15), to be amended in the specification. The substitute specification filed must be accompanied by a statement that it contains no new matter. Such statement must be a verified statement if made by a person not registered to practice before the Office.

In addition, the substitute specification mailed 10-27-00 (Paper No. 14), has not been entered because it does not conform to 37 CFR 1.125(b) because: there is no statement of new matter and because there is no marked up copy of the original specification provided. Therefore, the objection to the specification has been maintained.

Applicants attach to this response a statement confirming that the substitute specification submitted on 27 October, 2000 contained no new matter and a marked up copy clearly showing the designated changes made to the original specification.

35 U.S.C. § 112, first paragraph, enablement requirement.

Claims 2-4, 6 and 13 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

There is insufficient guidance in the instant specification and in the prior art for an antigen presenting vesicle free from its natural surroundings obtainable from an antigen presenting cell comprising a membrane and an MHC Class I protein or fragment thereof, as recited in claim 13, and its dependent claims 2-4 and 6, though the specification, is enabling for an antigen presenting vesicle free from its natural surroundings obtainable from an antigen presenting cell comprising a membrane and an MHC class II protein or fragment thereof. As evidenced by Figure 1 in Delves et al., (Molecular Medicine Today, 3(2):55-60, 1997), it is well known in the art that MHC Class I and Class II antigen presentation follow distinct, compartmentalized pathways, and that Class II bind antigens in endosomes or specialized loading compartments that are distinct from Class I molecules. Therefore, it is not clear from the instant specification that it is possible to isolate exosomes comprising class I (as opposed to class II) molecules from the supernatant of antigen presenting cells as taught by the specification.

Based upon the paucity of information contained within the instant specification in this regard, it would require an undue amount of experimentation on the part of one skilled in the art to use the claimed polypeptide for the asserted utilities.

In view of the quantity of experimentation necessary to use the claimed invention, the lack of working examples, the unpredictability of the art, the lack of sufficient guidance in the specification, it would require an undue amount of experimentation on the part of one skilled in the art to use the claimed methods for the asserted utilities, and this is not sanctioned by the statute.

Though applicant's arguments and amendments in their amendment mailed 10-27-00 (Paper No. 15), have been carefully considered, the 112 first rejection is maintained essentially for the reasons of record and is repeated below [above] for applicant's convenience. Applicants traverse the rejection on the grounds that the methods of differential centrifugation and sucrose gradients were well known at the time of the priority date of the applicant's invention with which the examiner agrees. The examiner also agrees with applicant that it was well known at the time of the priority date of the applicant's invention, that techniques for specifically obtaining Golgi or ER enriched fractions which contain MHC Class I proteins, were also well known. The examiner also agrees that the instant specification have described methods of differential centrifugation and isolation of subcellular fractions over sucrose gradients, but the examiner notes that the examples of an antigen presenting vesicle in the instant specification asserted by applicant all refer to the isolation of MIIC and exosomes, and not subcellular fractions which are not vesicles. Further, though said MIIC and exosome vesicles were known to contain MHC Class II as disclosed in the instant specification and as was well known in the art at the time of the invention, neither the applicant's specification nor the art at the time the invention was made, taught or disclosed that the vesicles of exosomes or MIIC contain class I proteins as evidenced by Zitvogel et al (Nature Medicine 4(5):594-600, May 1998. Zitvogel et al teach that it was surprising that human monocyte derived multivesicular MIICs contain abundant MHC class I molecules and further that multilamellar MIICs did not contain MHC Class I proteins(see entire article, especially page 594, column 2). Zitvogel et al further teach that it was unexpected that multivesicular late endosomes and exosomes in dendritic cells bear MHC Class I molecules(see entire article, especially page 598, column 2, middle paragraph). In the virtual absence of guidance from the instant specification of which vesicles contain class I proteins at the time the invention was made, and in the absence of a working definition of vesicle in the instant specification, it it would require an undue amount of experimentation on the part of one skilled in the art to use the claimed polypeptide [sic] for the asserted utilities. Furthermore Zitvogel et al teach that the potential advantages of exosomes in immunotherapy is due to its high levels of peptide bound MHC Class I and Class II molecules (see last paragraph of article) and the efficacy of the claimed vesicles with only class I and not class II molecules in immunotherapy is not clear.

Applicants respectfully traverse the rejection of Claims 3-4, 6 and 13 under 35 USC §112, first paragraph, because the specification teaches the skilled artisan how to make and to use an antigen presenting vesicle comprising an MHC class I protein. Furthermore, the Examiner is thanked for bringing Zitvogel, *et al.* to Applicants' attention because this manuscript demonstrates the operability of Applicants' invention.

Contrary to the Examiner's assertions, the instant specification provides detailed guidance on how to isolate exosomes from antigen presenting cells (page 2, line 25 through page 3, line 7 and page 8, line 23 through page 9, line 8 of the original specification), and demonstrates how to use them to stimulate T cells (page 10, lines 12-27 of the original specification). In lines 2-3 of the Abstract found on the cover page of the originally filed

specification, it is stated that "Exosomes are vesicles derived from MHC class II enriched compartments in antigen presenting cells. The exosomes possess MHC II and/or MHC I molecules at their surface and possibly peptides derived from processed antigens in said MHC's." Additionally, on page 6, lines 3-4 of the original specification, it is stated that "These vesicles preferably will contain major histocompatibility complex (MHC) I and/or II...". Although the claimed antigen presenting vesicles are exemplified by identifying the presence of MHC class II molecules, the specification teaches the presence of MHC class I molecules on multivesicular MIICs. The post-filing publication of Zitvogel, *et al.* confirms the operability of the claimed antigen presenting vesicles, and that multivesicular MIICs can comprise MHC I and/or MHC I molecules. Independent Claim 13 was amended in the response filed on 27 October, 2000 to recite an antigen presenting vesicle comprising a MHC class I molecule in order to address the restriction requirement imposed by the Examiner (paper 10). However, the specification explicitly teaches that the exosomes (which are vesicles) possess MHC II and/or MHC I molecules at their surface.

The Zitvogel, *et al.* publication further confirms the operability of the specific parameters of differential centrifugation and fractionation in linear sucrose gradients taught in the instant specification (page 2, line 25 through page 3, line 7; page 8, line 23 through page 9, line 8; and page 11, line 36 through page 12, line 14 of the original specification) for isolating antigen presenting vesicles that comprise MHC I and/or MHC II molecules. The Zitvogel, *et al.* publication confirms that *no experimentation* is necessary, because the differential centrifugation and fractionation over a linear sucrose gradient taught in the instant specification can be used to isolate exosomes that comprise MHC I and/or MHC II molecules with a reasonable expectation of success. Western blot and immunoprecipitation analyses to detect the presence of MHC I molecules as well as MHC II molecules can be carried out using techniques well known to those in the art at the time of filing of the application, and by following the procedures taught in the instant specification (page 3, line 1 through page 4, line 11; page 8, line 4 through page 10, line 9; and page 12, line 31 through page 13, line 13 of the original specification), but using anti-MHC class I antibodies readily available to those in

the art (*see* attached abstracts from Scott and Dawson (1995) *J Immunol* 155:143; Kaufman, *et al* (1995) 92:6484; Atta, *et al* (1995) *Clin Exp Immunol* 101:121; Khilko, *et al* (1995) *J Immunol Methods* 183:77; Rangel, *et al* (1995) 6:195).

The work of Zitvogel, *et al* demonstrates the correctness of Applicants assertion that "the exosomes possess MHC II and/or MHC I molecules at their surface" and that the detailed differential centrifugation and fractionation procedures taught in the instant specification apply to the isolation of antigen presenting vesicles that comprise MHC I and/or MHC II molecules. In view of the foregoing, Applicants respectfully assert that the instant specification properly enables those of ordinary skill in the art to make and to use an antigen presenting vesicle comprising an MHC I molecule. Accordingly, the Examiner is respectfully requested to withdraw the rejection of Claims 3-4, 6 and 13 under 35 USC §112, first paragraph.

35 U.S.C. § 112, first paragraph, written description requirement.

> **Claim 13 and dependent claims 2-4 and 6 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. There is no support in the specification or claims as originally filed for the recitation of "wherein said antigen presenting vesicle is obtainable from a cell." (Underline added) as recited by the instant claims. There appears to be support for the recitation of an antigen presenting cell as originally filed. There is no written description of the claimed invention in the specification or claims as originally filed. Thus the claimed invention constitutes new matter.**

In accordance with the Examiner's suggestion, Applicants have amended Claim 13 to recite "an antigen presenting cell", as originally filed.

35 U.S.C. § 112, second paragraph.

> **Claim 6 is rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.**
>
> **Claim 6 recites the limitation "said antigen presenting cell" in reference to the cell recited in claim 13. There is insufficient antecedent basis for this limitation in the claim 13.**

This rejection is believed avoided by amending Claim 13 to recite "an antigen presenting cell".

35 U.S.C. § 102(b)

> **Claims 13, 2, 3, 4 and 6 are rejected under 35 U.S.C. § 102(b) as being anticipated by Amigorena et al (Nature, 369:113-120, 1994).**
>
> **Amigorena et al teach the subcellular fractionation of a B cell line to produce fractions containing membrane vesicles with MHC Class II molecules (see entire article, especially page 114, column 2, last paragraph), which contained processed peptide (see entire article, especially page 118, first paragraph of the Discussion Section). Therefore, the referenced teachings anticipate the claimed references. The open language of "comprising" is noted in claim 13.**
>
> **Though applicant's arguments and amendments in their amendment mailed 10-27-00 (Paper No. 15), have been carefully considered, the Amigorena 102(b) rejection is maintained essentially for the reasons of record and is repeated below [above] for applicant's convenience. Applicants traverse the rejection on the grounds that this reference does not teach or suggest antigen presenting vesicles comprising a membrane and an MHC Class I protein. However, in view of the post filing date teachings of Zitvogel et al that the class II containing MIIC vesicles also contain Class I proteins (see section 5 of this office action), the claimed functional limitations of said vesicles containing Class I proteins would be inherent properties of the referenced vesicles.**

Applicants respectfully traverse the rejection of Claims 3-4, 6 and 13 under 35 USC 102(b), because there is absolutely no disclosure in Amigorena, *et al* to teach or suggest an antigen presenting vesicle comprising a MHC class I molecule. A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. MPEP 2131. Furthermore, as pointed out in the previous response, the Examiner's distinction between antigen presenting vesicles comprising MHC class I molecules and antigen presenting vesicles comprising MHC class II molecules has been made of record (*see* page 4 of paper 10 and page 2 of paper 13). Unlike the instant specification, which teaches that "the exosomes possess MHC II and/or MHC I molecules at their surface" (*see* abstract), Amigorena, *et al.* make absolutely no mention of any kind regarding antigen presenting vesicles comprising a MHC class I molecule. The Amigorena manuscript does not even provide the skilled artisan with any indication that it would be useful to look for the presence of MHC class I molecules on antigen presenting vesicles. This is first taught by Applicants in the instant specification.

The Examiner has maintained this rejection based on the disclosure of Zitvogel, *et al.* that multivesicular MHC class II compartments (MIICs) from human and mouse bone marrow derived dendritic cells also contain MHC class I molecules. However, to establish a rejection

based on inherency, the extrinsic evidence must make clear that the missing descriptive matter is *necessarily present* in the thing described in the reference [emphasis added]. Inherency may not be established by probabilities or possibilities, and the mere fact that a certain thing may result from a given set of circumstances is not sufficient. MPEP 2112 and 2112.01. Amigorena, *et al.* disclose MIICs isolated from the murine A20 B-cell lymphoma cell line that only contain MHC class II molecules. Certainly, there is nothing in the Amigorena manuscript that even alludes to the possible presence of MHC class I molecules in MIICs isolated from mouse A20 B cells. The Zitvogel manuscript is concerned with exosomes from human and mouse bone marrow derived dendritic cells, but is not concerned with B cells and repeatedly correlates the finding of MHC class I molecules with exosomes obtained from human or mouse bone marrow derived dendritic cells. Applicants respectfully assert that the disclosure of Zitvogel, *et al.* regarding exosomes from bone-marrow derived dendritic cells that contain both MHC class I and MHC class II molecules *does not necessarily establish* that the MIICs from A20 B cells disclosed by Amigorena also must have MHC class I molecules. Therefore, it is respectfully submitted that the Examiner has not properly met the requirements set forth to establish anticipation based on inherency.

Because the Amigorena manuscript does not teach or suggest anything regarding MIICs with MHC class I molecules, and the requirements for a rejection based on inherency have not been met, Applicants respectfully submit that Amigorena, *et al* can not properly anticipate the claimed antigen presenting vesicles. Accordingly, the Examiner is respectfully requested to withdraw this rejection.

CONCLUSION

In view of the above amendment and remarks, it is submitted that this application is now ready for allowance. Early notice to that effect is solicited. If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned at (650) 328-4400.

Respectfully submitted,

Dated: 17 April, 2001

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APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: **CELL-DERIVED ANTIGEN PRESENTING VESICLES**

INVENTORS: JOHANNES J. GEUZE AND CORNELIS J.M. MELIEF

Technical Field

The invention relates to the field of immunology, especially the cellular responses of the immune system, more in particular to the induction of said responses by peptides presented in the context of major histocompatibility complexes I and/or II.

Background

It is known that antigen presenting cells take up antigens through endocytosis, whereafter these antigens are cleaved into peptides which are presented at the surface of said antigen presenting cells in the context of a major histocompatibility complex. By this presentation on the surface the peptides derived from the original antigen can be recognized by for instance helper T-lymphocytes, further activating the cellular immune response.

Thus Helper T-lymphocytes recognize exogenous antigens bound to major histocompatibility complex (MHC) class II molecules expressed by a variety of antigen presenting cells (APCs) such as β-lymphocytes, macrophages and dendritic cells (1). Compelling evidence indicates that newly synthesized α and β subunits of MHC class II in association with the invariant chain (I-chain) are transported to intracellular compartments before reaching the plasma membrane (2,3). In these compartments the [l]I [amendment made on 2 February, 1998]-chain is degraded and MEC class II are potentially free to bind antigenic peptides arising from the degradation of antigens internalized by the APC (1, 4). We and others have shown that most of the intracellular MHC class II molecules reside in a [lysosome] lysosome-like, MHC-class II-enriched compartment (MIIC) which contains characteristic membrane vesicles and concentrically arranged membrane sheets (5, 6, 7, 8, 9, 10). MIICs and the related CIIVs (11), likely represent the meeting point between MHC class II and antigenic peptides (8,12). Once loaded with peptide, MHC class II molecules are transferred to the cell surface via an unknown pathway for presentation to T-lymphocytes.

Electron microscopy of immunogold labeled ultra thin cryosections from several human B-lymphoblastoid cell lines revealed MIICs whose surrounding membrane was contiguous with the plasma membrane in an exocytotic fashion and showed extracellular vesicles reminiscent of those present in non-fused MIICs (Figure 1A and B). Similar secretion of vesicles, termed exosomes, has been described for reticulocytes (13). Exosomes from B cells immunolabeled for the [lysosomal] lysosomal membrane proteins LAMP1 (Figure 1B) and CD63 (not shown) known to be expressed in MIICs (5, 6). Both LAMP1 and CD63 were absent from the rest of the

plasma membrane. Scarce labeling for MHC class II was associated with the limiting membrane of the fused MIICs but MHC class II was enriched in the externalized exosomes (Figure 1A and B). To test the release of MIIC contents further, B cells were allowed to internalize 5 nm gold particles conjugated to Bovine Serum Albumin (BSAG), and were then washed and reincubated in the absence of BSAG. Exosomes associated with previously endocytosed BSAG began to appear in exocytotic profiles after 30 min of uptake (10 min pulse and 20 min chase) (Figure 1B) and were abundant after 50 min (10 min pulse and 40 min chase) (Figure 1A). We conclude that multivesicular MIICs of human B-cell lines can fuse with the plasma membrane thereby releasing MHC class II-rich exosomes into the extracellular milieu.

Description of the Related Art [text for references entered from page 11, line 3 through page 15, line 16 of the original specification]

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2. J. J. Neefjes, V. Stollorz, P. J. Peters, H. J. Geuze, H. L. Ploegh, *Cell* 61, 171-183 (1990). [page 11, lines 8-9]

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19. K. Guy, V. Van Heyningen, B. B. Cohen, D. L. Deane, C. M. Steel, *Eur. J. Immunol.* 12, 942-948 (1982). [page 13, lines 14-15]

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29. C. V. Harding, H. J. Geuze, *J. Cell Biol.* 119, 531-542 (1992). [page 15, lines 9-10]

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Brief Description of the Drawings [text for figures entered from page 8, line 3 through page 10, line 27 of the original specification]

Figure 1:

MIICs are exocytotic compartments. T2-DR3 cells were incubated in the presence of 5 nm BSAG for 10 min., washed, chased for 40 min. and processed for cryoultramicrotomy as described (30). Ultrathin cryosections were immunolabeled with a rabbit polyclonal anti-class II antibody (5) and antibody binding sites were visualized with protein A conjugated to gold (PAG with sizes in nm indicated on the figures). MHC class II labeling is present at the limiting membrane of the exocytotic profile and on the exosomes. The profile also contains abundant re-externalized BSAG particles. PM: plasma membrane. B, RN cells were pulsed with BSAG for 10 min. and chased for 20 min. Ultrathin cryosections were double-immunolabeled with anti-class II antibody and with a monoclonal anti-LAMP1 antibody (31) as indicated. One of two neighboring profiles is shown, exocytotic profile containing BSAG and numerous exosomes labeled for MHC class II and LAMP1. Bars, 0.1 μm.

Figure 2:

Isolation of exosomes from cell culture media. A, RN cells were washed by centrifugation and re-cultured in fresh medium for 2 days. Cell culture media (35 ml) containing 2-5 $x10^8$ RN cells were centrifuged twice for 10 min. at 300 g (lane 1, first run; lane 2, second run). Lane 1 contains material from 0.6 x 10^6 cells. Membranes in the culture medium from 2-5 x 10^8 cells were pelleted by sequential centrifugation steps: twice at 1200 x g (lane 3 and 4), and once at 10,000 x g (lane 5), 70,000 x g (lane 6) and 100,000 x g (lane 7). The pellets were solubilized at 100°C under reducing conditions and analyzed by Western blotting using [^{125}I]-protein A. Per lane, samples equivalent to 1 x 10^6 cells were loaded. MHC class II α and β chains were recovered mainly from the cells (lane 1) and from the 70,000 x g pellet (lane 6). B, whole mount electron microscopy of the 70,000 x g pellet immunogold labeled for MHC class II. The 70,000 x g pellet was resuspended in RPMI medium, adsorbed to Formvar-carbon coated EM grids, fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer, immunolabeled with rabbit polyclonal anti-class II antibody and 10 nm PAG and stained using the method described for ultra-thin cryosections (30). The pellet is composed of 60-80 nm vesicles showing abundant MHC class II labeling. Bar, 0.2 μm

Figure 3:

Figure 3A, MHC class II present in the media are membrane bound. Membranes pelleted from culture media at 70,000 x g after differential ultracentrifugation were fractionated by floatation on sucrose gradients, and the non-boiled and non-reduced fractions analyzed by SDS-PAGE and Western blotting with the rabbit polyclonal anti-class II antibody (17). MHC class II molecules were recovered in fractions 5 to 12 corresponding to densities of 1.22-1.10 g/ml. The majority of MHC class II was in the SDS-stable compact form with a MW of 56-60 kD (Coc/β).

Figure 3B, Release of newly synthesized MHC class II molecules. RN cells were pulse-labeled with [^{35}S] methionine for 45 min. (lane 0) followed by chases in the absence of label for 6, 12 and 24 hours. MHC class II molecules were immunoprecipitated from lysates of the cells and pelleted exosomes with the monoclonal DA6.231 anti-class II antibody. RN cells (2×10^8) were washed 3 times with ice cold PBS and incubated for 30 min. at 0°C with 1mg/ml Sulfo-NHS-biotin (Pierce). Biotin was quenched for 30 min. with 50 mM NH_4Cl. After washing with ice cold PBS, half of the cells were solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. The remaining biotinylated cells were homogenized. The homogenates were centrifuged and ultracentrifuged identically to the cell culture supernatants and the 70,000 x g pellets solubilized in SDS-sample buffer supplemented with β-mercaptoethanol (control for plasma membrane remnants). Exosome preparations (70,000 x g pellets of cell culture media from 2×10^8 cells) were biotinylated as described above and solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. MHC class II was immunoprecipitated from a sample of biotinylated exosomes with the monoclonal anti-class II antibody DA6.231 (19). The biotinylated cell membranes, biotinylated exosomes and immunoprecipitated MHC class II were analyzed by SDS-PAGE and Western blotting with ^{125}I-Streptavidin. Immunoprecipitated MHC class II molecules were dissociated from the sepharose beads at non-reducing conditions at room temperature and analyzed by SDS-PAGE and fluorography. After pulse-labeling (0), MHC class II immunoprecipitated from the cells as SDS-unstable complex of α-β-invariant chain. SDS-stable α-β dimers were recovered from the cells after 6 hours of chase and the signal increased thereafter. In the exosomes pellets SDS-stable αβ dimers started to appear at 12 hours. Figure 3C, Exosomes and plasma membrane display different patterns of biotinylated proteins. In plasma membranes (lane 2) and

experimentally produced remnants of plasma membranes many biotinylated proteins are detected with ^{125}I-Streptavidin (lane 5). In exosomes (lanes 3 and 4, show increasing concentrations of exosomes, respectively) two major proteins with a MW of 60-70 kD are detected. Lane 1 shows the immunoprecipitation of biotinylated class II α and β chains from exosome lysates. In these assay the higher electrophoretic mobility of α and β chains is due to their efficient binding to biotin. Two minor bands at a MW of 200-300 kD are detected in exosomes (lanes 1, 3 and 4, arrows) and are absent from the plasma membrane.

Figure 4:

Presentation of HSP 65 antigen by HLA-DR15 positive RN B cells and exosomes to the CD4$^+$ T cell clone 2F10. Proliferative responses to naive cells Figure 4A, to cells pre-incubated with antigen Figure 4B, to exosomes derived from naive cells Figure 4C and to exosomes derived from cells pre-incubated with antigen (D). The closed symbols show proliferation measurements after addition of HSP 65 derived peptide (418-427), the open symbols where peptide was not added. HLA-class II restriction was determined by adding 10 µg/ml anti-DR antibody (triangles), anti-DP (circles), or no antibody (squares). The exosomes at the highest concentration were derived from media of 1.6 x 10^6 cells. All assays were performed in triplicate and results are expressed in cpm [^{3}H]-thymidine incorporated into T cells. The SEM for triplicate cpm measurements was less then 10%. Results shown form a representative example of experiments performed in [duplo, amendment made on 2 February, 1998] duplicate.

Description of the Preferred Embodiments

For a further characterization, exosomes were isolated from the culture media of the human B cell line RN by differential centrifugation (Figure 2). Pelleted membranes were analyzed by [DS] SDS [amendment made on 2 February, 1998]-PAGE and Western blotting. After removal of cells, the majority of MHC class II-containing membranes sediment at [70.000 g] 70,000 x g (Figure 2A, lane 6). The [70.000 g] 70,000 x g pellets were composed of a homogeneous population of vesicles labeled for MHC class II (Figure 2B). The vesicles were morphologically similar to those present in MIICs and in exocytotic profiles of sectioned cells (Figures 1A and B): their size ranged from 60 to 80 nm. To obtain biochemical evidence that the secreted MHC class II is membrane bound, [70.000 g] 70,000 x g pellets were fractionated by floatation in linear sucrose gradients [(14)]. [text inserted from note 14 on page 11, line 36

through page 12, line 12 of the original specification] The 70,000 x g pellet obtained after differential centrifugation of the cell culture supernatants of RN B lymphoblastoid cells was resuspended in 5 ml of 2.5 M sucrose, 20 mM Hepes/NaOH pH 7.2. A linear sucrose gradient (2 M-0.25 M sucrose, 20 mM Hepes- NaOH, pH 7.2) was layered over the exosome suspension in a SW27 tube (Beckman) and was centrifuged at 100,000 x g for 15 hrs. Gradient fractions (18 x 2 ml) were collected from the bottom of the tube, diluted with 3 ml PBS and ultracentrifuged for 1 hr at 200,000 x g using a SW50 rotor (Beckman). The pellets were solubilized at room temperature in SDS-sample buffer lacking -mercaptoethanol and analyzed by SDS-PAGE and Western blotting using ^{125}I-Protein A.

[new paragraph] Western blot analysis of the non-boiled and non-reduced gradient fractions showed that MHC class II molecules floated to an equilibrium density of 1.13 g/ml, confirming their association with membrane vesicles (Fig. 3A). MHC class II molecules recovered from the gradient fractions were predominantly in the SDS-stable, compact form indicating their stabilization by bound peptides (15). Together, these results show that the secreted MHC class II is associated with membrane vesicles and has bound peptides.

[new paragraph] To determine the kinetics and the extent to which newly synthesized MHC class II molecules are released into the medium, RN cells were metabolically pulse-labeled for 45 min. with [^{35}S]-methionine and chased for up to 24 hours in the absence of label [(16)]. [text inserted from note 16 on page 12, lines 16-29 of the original specification] RN cells were pulsed for 45 min. with 50 Mbq/ml [^{35}S]- methionine (Tran-Slabel, ICN, CA) and chased for different periods of time ($5x10^7$ cells per time point). After pulse-chase labeling, the cells were pelleted by centrifugation for 10 min. at 300 x g. The supernatants were collected and centrifuged for 5 min. at 10,000 x g and then for 30 min. at 200,000 x g in a SW60 rotor (Beckman). Cells and the 200,000 x g pellets were lysed and MHC class II and TfR were immunoprecipitated from equal samples of the lysates. TfR was immunoprecipitated as described previously (16). MHC class II was quantitated using a Phosphoimager. After pulse-labeling MHC class II was immunoprecipitated as SDS-unstable α-β-[I] I-chain complexes (Fig. 3B, lane 0). At 6 hours of chase part of MHC class II molecules were converted to SDS-stable, α-β-peptide complexes consistent with the kinetics reported for other human B cell lines (2, 17). Recovery of [^{35}S]-compact MHC class II from pelleted exosomes started at 12 hours and amounted 10 + 4% (n=5) of the total newly synthesized MHC class II after 24 hours of chase. The relatively slow rate by which newly synthesized MHC class II was secreted into the

medium suggests that insertion from the limiting membrane of MIICs into the plasma membrane during exocytosis is probably not the only pathway by which MHC class II molecules are delivered to the cell surface.

[new paragraph] To test the possibility that the vesicles recovered from the medium represented shed plasma membrane fragments or cell debris instead of exosomes, cells and exosome preparations were [biotinilated] biotinylated and the patterns of the [biotinilated] biotinylated proteins were studied by Western blotting with ^{125}[I] I-Streptavidin [(18)]. [text inserted from note 18 on page 12, line 31 through page 13, line 13 of the original specification] RN cells (2 x 10^8) were washed 3 times with ice cold PBS and incubated for 30 min. at 0°C with 1 mg/ml Sulfo-NHS-biotin (Pierce). Biotin was quenched for 30 min. with 50 mM NH_4Cl. After washing with ice cold PBS, half of the cells were solubilized in SDS-sample buffer supplemented with β-mercaptoethanol. The remaining biotinylated cells were homogenized. The homogenates were centrifuged and ultracentrifuged identically to the cell culture supernatants and the 70,000 x g pellets solubilized in SDS-sample buffer supplemented with β-mercaptoethanol (control for plasma membrane remnants). Exosome preparations (70,000 x g pellets of cell culture media from 2 x 10^8 cells) were biotinylated as described above and solubilized in SDS-sample buffer. supplemented with β-mercaptoethanol. MHC class II was immunoprecipitated from a sample of biotinylated exosomes with the monoclonal anti-class II antibody DA6.231 (19). The biotinylated cell membranes, biotinylated exosomes and immunoprecipitated MHC class II were analyzed by SDS-PAGE and Western blotting with ^{125}I-Streptavidin.

Figure 3C reveals differential patterns of [biotinilated] biotinylated proteins in exosomes and plasma membranes. Whereas plasma membranes show a broad spectrum of [biotinilated] biotinylated proteins (Figure 3C, lane 2), two proteins are enriched in exosomes (Figure 3C, lanes 3 and 4). Immunoprecipitation of the [biotinilated] biotinylated exosomal proteins with a monoclonal anti-class II antibody (19) identified these proteins as MHC class II (α and β subunits (Figure 3C, lane 1). Furthermore, the exosomes contain two minor bands at higher molecular weight which are not clearly detected in plasma membranes (Figure 3C, lanes 3 and 4). These proteins were also immunoprecipitated with the anti-class II antibody (Figure 3C, lane 1). To test the unlikely possibility that plasma membrane fragments eventually present in the 70,000 x g pellets contributed to the enrichment of MHC class II in exosomes, [biotinilated] biotinylated cells were homogenized and the homogenates were processed as the cell culture

supernatants [(18)], as described above. Very low amount of membranes are pelleted at 70,000 x g and these show a pattern of [biotinilated] biotinylated proteins matching that of total plasma membrane, as expected (Figure 3C, lane 5). When the cells were metabolically labeled with [^{35}S]-methionine for 45 min. and chased for up to 24 hours [(16)], as described above, the [^{35}S]-Transferrin receptor (TfR) ([^{35}S]-TfR) did not appear in exosomes at any chase time (data not shown). TfR is present at the plasma membrane of B cells but is absent from MIIC (8, 10). Together, these observations emphasize that exosomes are not derived from shed plasma membranes but represent an unique population of MHC class II- enriched membrane vesicles.

[text inserted from note 20 on page 13, lines 16-19] The internal MIIC vesicles are formed by inward budding of the limiting membrane of MIICs (*see* figures 16 and 17 in reference (6) similar to the process described for multivesicular bodies in other cell types (20)). Since the luminal domain of MHC class II molecules is exposed at the outside of exosomes (20), exosomes may be able to present antigens to T cells. To test this hypothesis, isolated exosomes were allowed to bind peptide 418-427 from the model antigen HSP 65 of *Mycobacterium Leprae*. The exosome preparations were then added to the T cell clone 2F10 which recognizes this peptide in the context of HLADR15 (21). In a parallel experiment, RN cells were allowed to endocytose HSP65 protein continuously for 24 hrs, washed, and incubated in the absence of antigen for another 24 hrs [(22)]. [text inserted from note 22 on page 13, line 26 through page 14, line 15 of the original specification.] The EBV-B cell lines RN (HLA-DR 15+) and JY (HLA-DR15-) were incubated in the presence or absence of purified HSP 65 protein from *Mycobacterium Leprae* (50 μg/ml) (22) for 4 hr in 10 ml serum free RPMI at 2 x 10^6 cells /ml, followed by the addition of 30 ml RPMI supplemented with 10% fetal calf serum (FCS) for 20 hr at 37°C. The cells were then washed to remove free antigen and incubated further for 24 hrs in RPMI/10% FCS medium at 37°C. Exosomes were prepared by differential centrifugation (Figure 2) and the efficiency of HSP 65 antigen presentation was measured by culturing 10,000 cells of the T cell clone 2F10 with irradiated (6000 rad) EBV cells. B cells or exosomes resuspended in 100 μl IMDM /10% pooled human serum were added to the T cell clone (50 μl IMDM /10% pooled human serum per well) in 96 well flatbottom microtiter plates (Costar, The Netherlands) for 4 days at 37°C, 5% CO_2 in humidified air. When indicated, 5 μg/ml of HLA-DR15 restricted epitope of HSP65 (peptide 418-427) was added to the exosomes. Sixteen hours before termination 0.5 μCi of [^{3}H]-thymidine was added to the wells. The cells were then harvested on glass fiber filters using an automatic cell harvester and the [^{3}H]-thymidine

incorporation into cell DNA was determined by liquid scintillation counting. The results are expressed as the mean of triplicate measurements).

[new paragraph] Both, exosomes incubated with antigenic peptide (Figures 4A and C) and exosomes derived from cells that were pre-incubated with antigen (Figures 4B and D) were able to induce a specific T cell response [(23)]. [text inserted from note 23 on page 14, lines 16-20 of the original specification] As a control, exosomes were prepared from culture media of an equivalent amount of DR15-negative JY cells that have been incubated or not with antigen. JY cells secreted an equivalent amount of exosomes but these were ineffective in stimulating T cell proliferation. A half maximal response was obtained with an amount of exosomes secreted by 3 x 10^5 RN cells in 24 hours (Fig. 4, D). In comparison $2x10^4$ intact RN cells were necessary to achieve the half maximal response (Fig. 4B[, 24]). [text inserted from note 24 on page 14, lines 21-24 of the original specification] From these data exosomes appear to be 16 times less efficient in antigen presentation. However, in antigen presentation assays contact between B and T cells may be more efficient due to sedimentation of cells. The responses observed were DR restricted. Anti-HLA-DR antibody blocked T cell proliferation completely, whereas anti-HLA-DP was ineffective (Figs 4B and D). From these data we conclude that culture media of B cells provide for a source of MIIC-derived microvesicles (exosomes) that can induce T cell responses by themselves [(25)]. [text inserted from note 25 on page 14, lines 25-28 of the original specification] Exosomes isolated from the culture medium of the murine B cell line TA3 (1-E^{k+}) incubated in the presence a RNase-derived peptide (aa 90-105) were also capable of stimulating IL2 secretion by WA.23 cells.

Exocytosis of MIIC vesicles by B-lymphocytes is reminiscent of the exocytosis of the vesicles contained in the cytolytic granules of cytotoxic T-lymphocytes (CTLs) (26). Both MIICs and cytolytic granules have [lysosomal] lysosomal characteristics and contain internal membranes. The internal vesicles of cytolytic granules are exocytosed by the CTLs upon CTL-target cell interaction and presumably have a role in the killing of target cells (26). Whether B-cell exosomes also have an extracellular role *in vivo* remains to be established. It has been suggested that follicular dendritic cells acquire MHC class II molecules released from surrounding B cells by an unknown mechanism (27). It is worth studying the possibility that exosomes serve as carriers of MHC class II-peptide complexes between different cells of the immune system. Whether physiological APCs like dendritic cells and macrophages generate exosomes has to be studied [(28)]. [text inserted from note 28 on page 14, line 35 through page

15, line 8] A number of studies documented the presence of intact MHC class II molecules in 100,000 x g fractions from B cell culture media and their association of with membrane lipids (28). Our present observations shed new light on these data and suggest that the released MHC class II molecules were likely derived from secreted exosomes. However, secretion of [lysosomal] lysosomal contents by macrophages has been documented and macrophage tubular [lysosomes] lysosomes are rich in MHC class II and contain membrane vesicles (29). It can be speculated that *in vivo*, exosomes may function as transport vehicles for MHC class II-peptide complexes responsible for maintenance of long term T cell memory or T cell tolerance. Finally, since exosomes can easily be obtained and are capable of presenting antigens specifically and efficiently, it is worth exploring their usefulness as biological vehicles in immunotherapy.

The invention therefore provides an antigen presenting vesicle free from its natural surroundings obtainable from antigen presenting cells, such as B-cells, macrophages or dendritic cells, especially Langerhans cells of the epidermis.

These vesicles preferably will contain major histocompatiblility complex (MHC) I and/or II, most preferably loaded with a peptide derived from or corresponding to an antigen which can be processed by antigen presenting cells.

It has been tried before to produce similar vesicles synthetically, for instance in the form of liposomes, but these attempts have so far not been successful. Now that we have surprisingly found that there are counterparts of said liposomes in nature, these counterparts can of course be used in any intended application of said liposomes.

The major advantage of the vesicles according to the invention is of course that they will automatically comprise all the necessary elements for antigen presentation. Further analysis of the vesicles, once discovered will therefore result in a better understanding of which elements are essential for said presentation on said vesicles. It will then of course be possible to arrive at vesicles according to the invention in other ways then by isolation from cells. The invention therefor does encompass all antigen presenting vesicles which comprise the essential elements for presenting such antigens, regardless of the way they are produced or obtained.

One may for instance think of synthetically prepared liposomes, provided with at least biologically active parts of (recombinant) MHC I or II, optionally provided with processing agents for antigens to be presented in the context of said MHC. Of course cells which produce these vesicles can also be provided with recombinant MHC I or II encoding genes, so that the desired MHC's will be present on the eventually resulting vesicles, etc.

[abstract entered from the cover page of the WO 97/05900 PCT publication of the instant application]

ABSTRACT

The invention provides a novel vehicle for vaccination, in particular peptide vaccination. The new vehicle has been termed an exosome. Exosomes are vesicles derived from MHC class II enriched compartments in antigen presenting cells. The exosomes possess MHC II and/or MHC I molecules at their surface and possibly peptides derived from processed antigens in said MHC's. Thus the exosome is a perfect vaccination vehicle in that it presents the peptide in a natural setting. The peptides present in the exosome in the MHC molecule may be processed by the antigen presenting cell from which the exosome is derived. Empty MHC molecules on exosomes may also be loaded with peptides afterwards.

Although vesicles which present peptides in the context of MHC I or II are preferred, it is also very useful to produce vesicles which do have the MHC's on their surface, but without a peptide being present therein. These vesicles can then be loaded with desired peptides having the right binding [motiv] motif [amendment made on 2 February, 1998] to fit in the respective MHC.

The first and perhaps foremost use of these vesicles that comes to mind is of course mimicking their role in nature, which is the presentation of peptides as antigens, for the stimulation of for instance T-cells. Thus the vesicles according to the invention can be very suitably used in for instance vaccines. These vaccines can be designed to elicit an immune response against any proteinaceous substance which has peptide antigens that can be presented in the context of MHC.

The vaccines may of course comprise suitable adjuvants, if necessary, carriers, if necessary, [ecxipients] excipients for administration, etc.

The vaccines can be used in the treatment or prophylaxis of many disorders, such as infections, immune disorders, malignancies, etc.

Very important applications will of course be the treatment or prophylaxis of AIDS, eliciting immune responses against tumors and the like.

Another important application of the vesicles according to the invention is that they may be used to induce tolerance to certain antigens, for instance by giving large doses of the vesicles orally.

Based on the description of the invention and specifically referring to the following experimental part illustrating the invention the person skilled in the art will be able to find further uses of the vesicles according to the invention without departing from the spirit of the invention.

[delete text on pages 8-15 because it has been inserted into the text on pages 1-7 as designated above.]

ANTI-CLASS I Abs

1: J Immunol 1995 Jul 1;155(1):143-8

MHC class I expression and transport in a calnexin-deficient cell line.

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The human leukemic cell line, CEM, and an NK-resistant variant of CEM, called CEM-NKR, were analyzed for protein differences by two-dimensional gel electrophoresis. One protein was found to be completely absent in CEM-NKR. This protein has been identified as calnexin. CEM-NKR also completely lacks calnexin RNA. Calnexin is thought to act as a molecular chaperone by assisting in the assembly and/or retention of MHC class I and many other membrane and secreted proteins. The surface expression of class I molecules on CEM-NKR was compared with CEM by several Abs. There was no significant class I expression differences between CEM and CEM-NKR using w6/32 (a conformational and beta 2-microglobulin-dependent mAb), HC-10 (a conformational and beta 2-microglobulin-independent mAb), and an anti-class I antiserum that reacts with native and denatured class I. The transport rate of class I in both cell lines was examined by pulse-chase experiments, immunoprecipitating class I with w6/32 and anti-class I antiserum. The results show that class I molecules in the calnexin-deficient cell line and its parent cell line are transported at similar rates. These results indicate that calnexin is not absolutely required for the viability of CEM or the transport and surface expression of human MHC class I molecules.

PMID: 7602092   [PubMed - indexed for MEDLINE]

ANTI-CLASS I Abs

1: Clin Exp Immunol 1995 Jul;101(1):121-6

Enhanced expression of MHC class I molecules on cultured human thyroid follicular cells infected with reovirus through induction of type 1 interferons.

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Certain viruses are known to modulate the cellular expression of MHC molecules. We have investigated whether reovirus types 1 or 3 can alter the normal MHC molecule expression on cultured human thyroid follicular cells (TFC). Primary TFC cultures were established from eight human thyroid donors and MHC class I and II expression was assessed by indirect immunofluorescence microscopy. Both types of reovirus enhanced MHC class I expression on TFC from all thyroid donors. Class II MHC protein was strongly induced by type 1 reovirus on TFC from one donor, while weak induction of expression, by either reo-1 or reo-3 virus, was noted on the TFC of five other donors. Studies on the mechanism(s) of MHC class I hyperexpression showed that mouse MoAb against the type 3 reovirus haemagglutinin (anti-HA3) reduced the ability of the virus to induce hyperexpression of class I MHC molecules on TFC. However, supernatant harvested from type 3 reovirus-infected TFC cultures maintained its ability to enhance class I expression after incubation with anti-HA3. Moreover, adding rabbit anti-sera to interferon-alpha (IFN-alpha) or IFN-beta inhibited the increased class I MHC expression on TFC by both types of reovirus. These data suggest that reoviruses (types 1 and 3) can enhance MHC class I on cultured TFC. The mechanism of MHC class I enhancement is most probably through the release of IFN-alpha and IFN-beta.

PMID: 7621581   [PubMed - indexed for MEDLINE]

ANTI-CLASS I Abs

1: J Immunol Methods 1995 Jun 14;183(1):77-94

Measuring interactions of MHC class I molecules using surface plasmon resonance.

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To examine the molecular interactions between major histocompatibility complex (MHC)-encoded molecules and peptides, monoclonal antibodies (mAbs), or T cell receptors, we have developed model systems employing genetically engineered soluble MHC class I molecules (MHC-I), synthetic peptides, purified mAbs, and engineered solubilizable T cell receptors. Direct binding assays based on immobilization of one of the interacting components to the dextran modified gold biosensor surface of a surface plasmon resonance (SPR) detector have been developed for each of these systems. The peptide binding site of the MHC-I molecule can be sterically mapped by evaluation of a set of peptides immobilized through the thiol group of cysteine substitutions at each peptide position. Kinetic binding studies indicate that the MHC-I/peptide interaction is characterized by a low to moderate apparent kass (approximately 5000-60000 M-1 s-1) and very small kdis (approximately 10(-4)-10(-6) s-1) consistent with the biological requirement for a long cell surface residence time to permit engagement with T cell receptors. Several mAb directed against different MHC-I epitopes were examined, and kinetic parameters of their interaction with MHC molecules were determined. These showed characteristic moderate association rate constants and moderate dissociation rate constants (kass approximately 10(4)-10(6) M-1 s-1 and kdis approximately 10(-2)-10(-4) s-1), characteristic of many antibody/protein antigen interactions. The interaction of an anti-idiotypic anti-TCR mAb with its purified cognate TCR was of moderate affinity and revealed kinetic binding similar to that of the anti-MHC mAbs. The previously determined interaction of a purified T cell receptor with its MHC-I/peptide ligand is characterized by kinetic constants more similar to those of the antibody/antigen interaction than of the MHC-I/peptide interaction, but is remarkable for rapid dissociation rates (apparent kdis approximately 10(-2) s-1). Such binding studies of reactions involving the MHC-I molecules offer insight into the mechanisms responsible for the initial specific events required for the stimulation of T cells.

PMID: 7602142   [PubMed - indexed for MEDLINE]

ANTI-CLASS I Abs

1: Eur Cytokine Netw 1995 May-Jun;6(3):195-202

Generation of memory CD4+, CD8+, CD45RO+ and CD16- lymphocytes activated with IL-2, INF-gamma, and TNF-alpha with specific cytotoxicity against autologous cervical cancer cells in a mixed leukocyte-tumour cell culture.

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Cytotoxic T lymphocytes (CTL) specific against autologous human cervical cancer cells were generated in vitro from peripheral blood leukocytes (PBL) from four patients with non-keratinized epidermoid carcinoma. For this purpose, these patients' PBL were co-cultured for 28 days either with IL-2 or a mixture of IL-2, IFN-gamma and TNF-alpha in the presence of autologous tumour cells (ATC). Our results showed that these CTL were highly cytotoxic for ATC, weakly cytotoxic for heterologous cervical cancer tumour cells, and not cytotoxic for carcinoma cell lines, normal cervix cells nor autologous PBL. Proliferation and cytotoxicity against ATC were greater when the PBL were activated with the three cytokines. These CTL had a CD4:CD8 ratio of 1:1, were CD16- and CD45RO+ and their killing activity was inhibited by antibodies against CD3, CD8 and MHC-class I but not by antibodies against CD4, CD16 or HLA-class II. The possibility of generating specific CTL in long term cultures for cervical cancer therapy is also discussed.

PMID: 8589277   [PubMed - indexed for MEDLINE]

ANTI-CLASS I Abs

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Immunology

Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I

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Communicated by Stanley G. Nathenson, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, March 22, 1995

ABSTRACT **Many studies have characterized the transmembrane signaling events initiated after T-cell antigen receptor recognition of major histocompatibility complex (MHC)-bound peptides. Yet, little is known about signal transduction from a set of MHC class I recognizing receptors on natural killer (NK) cells whose ligation dramatically inhibits NK cell-mediated killing. In this study we evaluated the influence of MHC recognition on the proximal signaling events in NK cells binding tumor targets. We utilized two experimental models where NK cell-mediated cytotoxicity was fully inhibited by the recognition of specific MHC class I molecules. NK cell binding to either class I-deficient or class I-transfected target cells initiated rapid protein tyrosine kinase activation. In contrast, whereas NK cell binding to class I-deficient targets led to inositol phosphate release and increased intracellular free calcium ($[Ca^{2+}]_i$), NK recognition of class I-bearing targets did not induce the activation of these phospholipase C-dependent signaling events. The recognition of class I by NK cells clearly had a negative regulatory effect since blocking this interaction using anti-class I $F(ab')_2$ fragments increased inositol 1,4,5-trisphosphate release and $[Ca^{2+}]_i$ and increased the lysis of the targets. These results suggest that one of the mechanisms by which NK cell recognition of specific MHC class I molecules can block the development of cell-mediated cytotoxicity is by inhibiting specific critical signaling events.**

Natural killer (NK) cells are $CD3^-$, $CD16^+$ lymphocytes that mediate lysis of certain tumor cells and virus-infected cells without prior sensitization (1). While NK cells are not major histocompatibility complex (MHC)-restricted in their killing of targets, numerous studies *in vitro* and *in vivo* have demonstrated that target cell expression of class I molecules can inhibit NK cell-mediated killing of these targets (2–7). However, attempts to define the basis for this negative regulation have been complicated by multiple factors. First, there appear to be several families of MHC-recognizing receptors on NK cells and within each family there are multiple members. Different receptors on NK cells also appear to recognize different subsets of MHC class I molecules (3). In addition, depending on the NK clone, MHC recognition can be alternatively activating or inhibiting (e.g., kp43) (8). Therefore, depending on the repertoire of MHC-recognizing molecules on a given subpopulation of NK cells and on the specific expression of MHC class I molecules on a given target, the nature of the effector–target interaction can be quite complex. Moreover, even among a clonal population of effector cells, there will likely be cells in differing states of activation. In the context of all of this heterogeneity, it has been observed that most cloned human NK cell lines are only partially inhibited in their killing of MHC-bearing targets. Indeed, Litwin *et al.* (9) examined >200 human NK cell clones and found few cases where there was an absolute inhibition of NK cell-mediated cytotoxicity. In this setting of "quantitative" rather than "qualitative" inhibition, experimentation has been unable to identify a discernible correlation between NK recognition of MHC and a reduction in early signaling events in the NK cells (10).

To more clearly assess whether MHC recognition by NK cells could directly alter proximal signaling events, we sought to identify experimental systems where there was a more absolute (i.e., qualitative) inhibition. First, we found that NKL, a $CD3^-$ interleukin 2 (IL-2)-dependent NK cell line that can effectively kill class I-deficient cells, was unable to kill specific HLA-B27-bearing targets. Similarly, certain cloned populations of NK cells bearing the $GL183^-/EB6^+$ phenotype are unable to kill HLA-Cw4-bearing targets (11). In each of these experimental models where MHC recognition fully inhibited NK cell-mediated cytotoxicity, proximal signaling in the NK cells was also inhibited. These results suggest that ligand binding by MHC-recognizing receptors on NK cells can alter transmembrane signaling and the subsequent development of the cytotoxic response.

MATERIALS AND METHODS

Cells. The NKL cell line was derived from the peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder (12, 13). Clonal human NK cell lines were isolated and passaged as described (14). The HLA class I-deficient C1R cell line and the HLA-transfected C1R sublines were generously provided by Peter Cresswell (Yale University). C1R cells express no detectable HLA-A molecules, low levels of HLA-B35, and normal levels of HLA-Cw4 (15).

Antibodies. The p58-specific monoclonal antibodies (mAbs) GL183 and EB6 were kindly provided by Alessandro and Lorenzo Moretta (Genoa, Italy) (16). The anti-phosphotyrosine antibody 1G2 was the kind gift of A. R. Frackelton, Jr. (Brown University). MB40.5 (reactive with conserved, α_2 domain-associated, monomorphic determinants on HLA-A, -B, and -C, IgG1) and G28-5 (anti-CD40, IgG1) were obtained from the American Type Culture Collection. All mAbs were purified from ascites using protein A-agarose affinity chromatography (Bio-Rad). $F(ab')_2$ fragments of MB40.5 were generated by digestion with immobilized pepsin (Pierce), and purity of the fragments was confirmed by SDS/PAGE followed by silver staining.

Measurements of Inositol Phosphates, Intracellular Calcium, Cytotoxicity, Conjugate Formation, and Protein Phosphorylation. Inositol phosphate release, intracellular free calcium concentrations ($[Ca^{2+}]_i$), cell-mediated cytotoxicity, con-

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PTK, protein-tyrosine kinase; MHC, major histocompatibility complex; NK, natural killer; mAb, monoclonal antibody; IL-2, interleukin 2; E:T, effector: target.

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jugate formation, and tyrosine phosphorylation were measured as described (10, 14, 17).

RESULTS

Class I Expression on Target Cells Inhibits NKL Cytotoxic Functions. The human cell line NKL was derived from the peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphocyte proliferative disorder (12). Functionally, NKL demonstrates cytolytic activity similar to normal peripheral blood NK cells in that it mediates natural killing of K562 and Fc receptor-dependent cytotoxicity of antibody-coated P815 cells (12). Since class I MHC molecules expressed on tumor targets can inhibit NK cell-mediated cytotoxicity, we chose to examine NKL for its ability to kill the class I-deficient C1R cell line compared to C1R transfected with various class I molecules (Fig. 1*A*). C1R cells expressing transfected HLA-B27 (C1R-B27) consistently demonstrated a near-absolute resistance to lysis by NKL. In contrast, C1R-A3, C1R-Aw68, C1R-B7, and C1R-Bw58 showed intermediate resistance to NKL (Fig. 1*A*), and C1R-A2 was killed just as well as untransfected C1R cells (data not shown). These data support previous observations that different class I molecules can vary in their ability to inhibit NK cell-mediated cytotoxicity. Since C1R-B27 mediated the greatest inhibition of NKL cells, we chose to focus on this interaction for subsequent studies.

To confirm that the decreased lysis of C1R-B27 was due to the expression of class I on the target cell surface, we pretreated the targets with $F(ab)'_2$ fragments of the anti-class I mAb MB40.5. Addition of MB40.5 increased lysis of C1R-B27 in a dose-dependent manner (Fig. 1*B*), whereas addition of the isotype-matched control mAb G28-5 against CD40 (also expressed on the targets) did not. Moreover, revertant sublines of class I-transfected C1R cells that had decreased surface class I expression showed increased susceptibility to NK cell-mediated killing (data not shown). Therefore, the resistance of C1R-B27 was due to its surface MHC class I expression. In addition, these results support the concept that MHC recognition by NK cells can confer a negative regulatory influence on the activation of these effector cells.

Recognition of HLA-B27 Expression Inhibits Inositol Phosphate Release and Elevated $[Ca^{2+}]_i$ in NKL Cells. To evaluate mechanisms for class I-mediated inhibition of NK cell-mediated cytotoxicity, we analyzed proximal signaling events in NKL cells stimulated with either C1R or C1R-B27. While C1R cells stimulated a 2- to 6-fold increase in the generation of inositol phosphates in NK cells, C1R-B27 stimulation of NKL did not result in significant inositol 1,4,5-trisphosphate (IP_3) release (Fig. 2*A*). Moreover, treatment of C1R-B27 with $F(ab')_2$ fragments of MB40.5 (anti-class I), but not G28-5 (anti-CD40), restored the ability of C1R-B27 to activate IP_3 production (Fig. 2*B*). Therefore, the increased lysis of anti-class I-treated C1R-B27 cells correlates with restoration of IP_3 production in NKL cells upon recognition of these targets.

Parallel findings were observed for elevation of $[Ca^{2+}]_i$ in NKL upon stimulation with these targets. Using indo-1 AM-labeled NKL cells and flow cytometry, we found C1R cells stimulated increased $[Ca^{2+}]_i$ in NKL, whereas C1R-B27 failed to initiate any detectable increase (Fig. 3). Treatment of C1R-B27 with anti-class I Ab restored the ability of C1R-B27 to induce increased $[Ca^{2+}]_i$ (Fig. 3). Addition of either anti-class I or anti-CD40 mAb to NKL cells alone or NKL cells stimulated with C1R cells had no effect on IP_3 release or calcium signaling (data not shown). Taken together, these results suggest that the inhibition of NKL activation after the

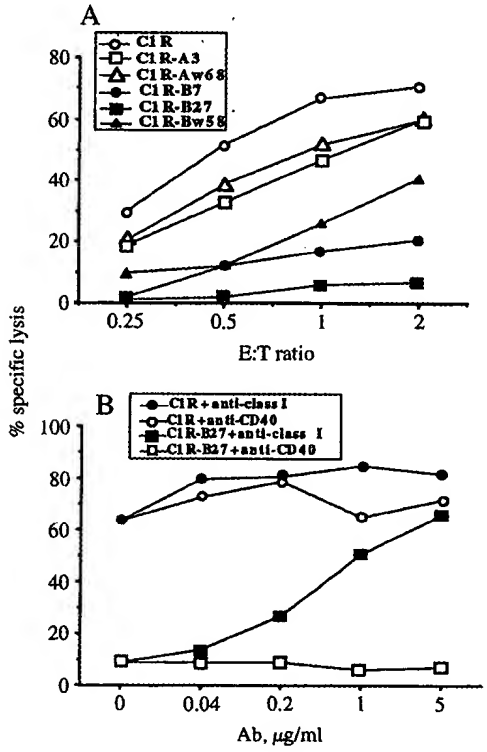

FIG. 1. HLA class I expression on target cells inhibits lysis by NKL cells. (*A*) NKL cells were used as effectors in a 4-hr ^{51}Cr release assay against class I-deficient C1R cells or stably transfected C1R cells expressing high levels of HLA class I molecules. (*B*) ^{51}Cr-labeled C1R cells or HLA-B27 transfected C1R cells (C1R-B27) were pretreated for 30 min with $F(ab')_2$ fragments of MHC class I-specific mAb (MB40.5) or an isotype-matched CD40-specific mAb (G28-5). NKL effector cells were then added at an effector:target (E:T) ratio of 1:1.

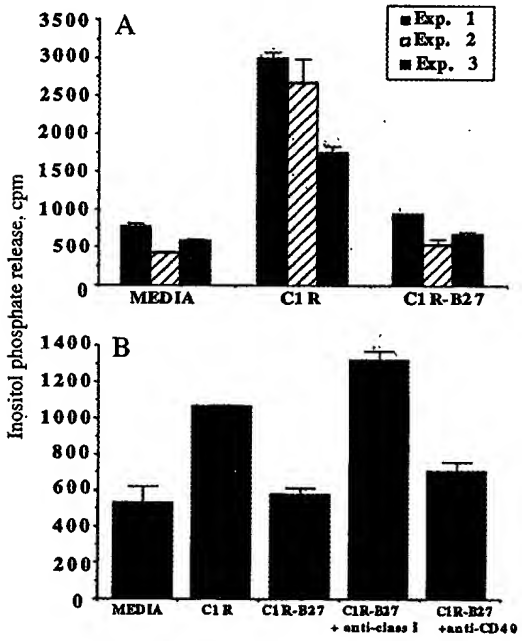

FIG. 2. Recognition of HLA-B27 expression on target cells inhibits inositol phosphate hydrolysis in NKL cells. (*A*) NKL cells were labeled with *myo*-[^{3}H]inositol, washed, and incubated for 30 min with medium alone, C1R cells, or C1R-B27 cells at an E:T ratio of 1:1. Total inositol phosphates were extracted, separated by anion-exchange chromatography, and quantitated by liquid scintillation spectroscopy. Three separate experiments, as indicated, showed similar results. Each bar represents the mean of triplicate samples in each experiment and the error bars indicate the SD. (*B*) C1R cells or C1R-B27 were pretreated with 10 μg of $F(ab')_2$ fragments of class I-specific or CD40-specific mAb per ml. Error bars represent the SD for triplicate samples. Data are representative of three separate experiments.

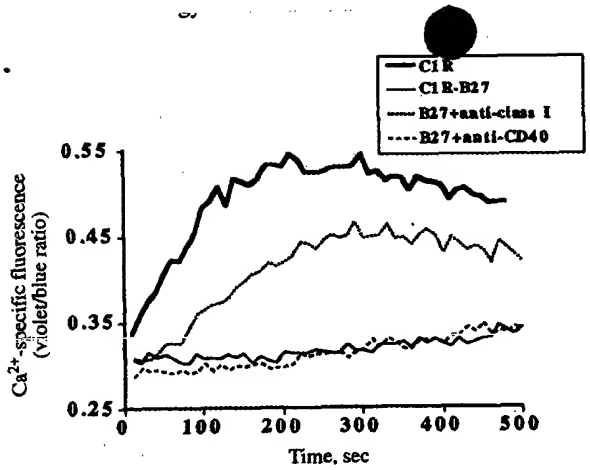

FIG. 3. Recognition of HLA-B27 expression on target cells inhibits calcium signaling in NKL cells. NKL cells were loaded with the calcium-sensitive dye indo-1 and stimulated with either C1R cells, C1R-B27 cells, or C1R-B27 cells pretreated with class I-specific or CD40-specific mAb. Changes in calcium-specific fluorescence were monitored by flow cytometry and expressed as the ratio of violet (390 nm) to blue (500 nm) fluorescence.

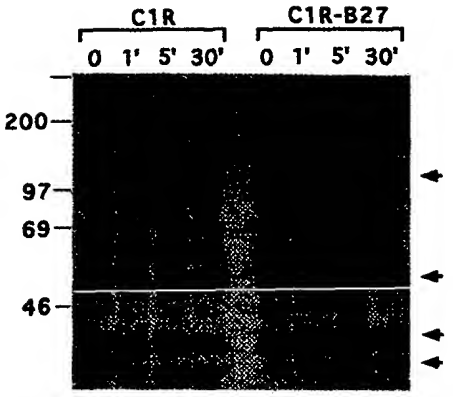

FIG. 4. C1R and C1R-B27 cells stimulate PTK activation in NKL cells. NKL cells were labeled with $^{32}P_i$, washed, and stimulated for the indicated time with either C1R or C1R-B27 cells at an E:T ratio of 1:2. Cells were then lysed and phosphotyrosine-containing proteins were immunoprecipitated with the mAb 1G2 linked to Sepharose. Lysates of unstimulated NKL cells (0 time) were mixed with lysates of an equivalent number of target cells to control for total protein content. Proteins were resolved on an SDS/8.5% polyacrylamide gel and transferred to an Immobilon-P membrane. Molecular masses (kDa) are indicated (left) and proteins with induced tyrosine phosphorylation are indicated by arrowheads (right).

recognition of HLA-B27 molecules is the result of an early block in production of phospholipase C (PLC)-derived second messengers.

Tyrosine Kinase Activation in NKL Cells Recognizing HLA-B27. Previously, our laboratory and others have demonstrated that protein-tyrosine kinases (PTKs) in NK cells provide early and requisite signals for PLC-γ activation and the subsequent development of cytotoxic function (17–21). Here we examined the influence of MHC recognition on the tyrosine phosphorylations induced by binding to susceptible targets. ^{32}P-labeled NK cells were incubated with either C1R or C1R-B27 cells, and cell lysates from these reaction mixtures were then immunoprecipitated with anti-phosphotyrosine antibody. Subsequent analysis by SDS/PAGE and autoradiography demonstrated that C1R cells and the HLA-B27 transfectants induced in NKL cells the rapid tyrosine phosphorylation of similar (based on identical molecular masses) substrates (Fig. 4). In each case, the indicated substrates were maximally phosphorylated 5 min after stimulation, and densitometric evaluation of substrates with induced tyrosine phosphorylation (bands indicated by arrowheads) showed a 2- to 4-fold increase in C1R-stimulated NKL cells and a 2- to 8-fold increase in C1R-B27-stimulated NKL cells. This result indicates that although HLA-B27 recognition can interrupt PLC-γ activation and the subsequent development of the cytotoxic response, certain proximal PTK-catalyzed signaling events are initiated. More broadly, this result further refutes the concept that MHC expression on target cells masks NK cell recognition of triggering epitopes on the targets (target interference model). Rather, MHC recognition itself appears to modulate a subset of the signaling events initiated after NK cell recognition of susceptible targets (effector inhibition model) (22).

Engagement of p58 Molecules Also Blocks PLC-γ-Mediated Activation Events. The p58 family of receptor molecules on NK cells appears to preferentially recognize certain HLA-C alleles (11, 23). Elegant studies have shown that interaction between HLA-C and p58 leads to inhibition of NK cell-mediated cytotoxicity, and blocking this interaction with mAbs against either the p58 molecules or HLA-C prevents the inhibitory interaction and leads to lysis of the target (11, 24). Here we sought to determine the nature of the p58-mediated inhibitory effect. For this purpose, we took advantage of the normal level of HLA-Cw4 on C1R cells. While the recognition of HLA-Cw4 has no inhibitory effect on the majority of NK cells, NK clones phenotyped as $GL183^-/EB6^+$ are potently inhibited after HLA-Cw4 recognition (Fig. 5*A* and ref. 11). Importantly, this inhibition is absolute and qualitative, much like NKL and HLA-B27. We examined $GL183^-/EB6^+$ NK clones for generation of PLC-mediated signals upon interaction with resistant C1R cells or cells susceptible to either natural cytotoxicity or Fc receptor-dependent cell-mediated killing (K562 or the anti-FcγRIII-producing hybridoma, 3G8, respectively) (Fig. 5*A*). Whereas K562 and 3G8 hybridoma cells stimulated inositol phosphate release and elevated $[Ca^{2+}]_i$, C1R cells (HLA-Cw4$^+$) failed to stimulate the PLC-γ-generated signals (Fig. 5 *B* and *C*). In contrast, C1R cells, which can be effectively

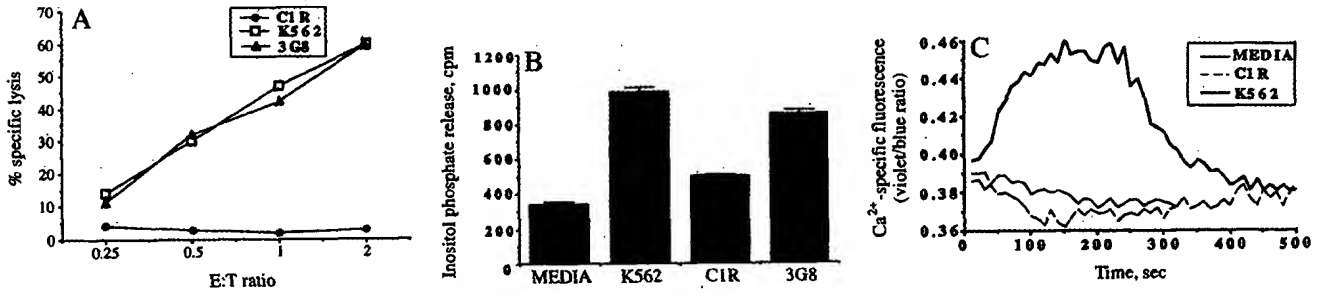

FIG. 5. Recognition of HLA-Cw4 expression on target cells inhibits the activation of $GL183^-/EB6^+$ NK cells. (*A*) $GL183^-/EB6^+$ cloned human NK cells were incubated with either C1R cells (HLA-Cw4$^+$), K562 cells, or anti-FcγRIII hybridoma cells (3G8) in a 4-hr ^{51}Cr release assay. Inositol phosphate hydrolysis (*B*) and calcium signaling (*C*) were separately analyzed for each effector–target combination. Results shown are representative of those obtained with five different $EB6^+$ NK clones.

killed by GL183$^-$/EB6$^-$ NK clones, did stimulate increased $[Ca^{2+}]_i$ and IP_3 release in GL183$^-$/EB6$^-$ NK cells (data not shown). These results are consistent with the notion that p58 recognition of HLA-Cw4 can prevent the generation of PLC-γ-derived second messengers.

Decreased Signaling After Recognition of MHC Class I$^+$ Targets Is Not Due to Decreased Conjugate Formation. The initial stage of NK cell-mediated cytotoxicity is the formation of stable conjugates between target and effector. One possible explanation for a lack of signaling events in NKL cells upon interaction with C1R-B27 would be if these cells did not effectively form conjugates. Although the presence of PTK activation after recognition of these class I-bearing targets suggests that functional effector–target conjugates are being formed, we sought, in separate analyses, to directly quantitate the influence of MHC expression on conjugate formation in this experimental system. Using two-colored flow cytometry, we found that C1R-B27 cells formed conjugates with NKL cells just as well as C1R cells (Table 1). Though we showed treatment of C1R-B27 with F(ab')$_2$ fragments of the anti-class I mAb, MB40.5, greatly increased NKL-mediated lysis (Fig. 1*B*), this treatment did not affect conjugate formation (Table 1). Additional studies of conjugate formation between EB6$^+$ NK clones and either C1R cells (resistant to lysis) or K562 (sensitive to lysis) also found no significant differences (Table 1). Therefore, the inhibition of proximal signaling events in NK cells interacting with class I$^+$ targets appears to follow normal conjugate formation.

Effects of Pretreatment with IL-2 on NK Cell Signaling and Cellular Cytotoxicity. Previously our laboratory and others have shown that for those NK cells that are partially inhibited in their killing of class I-bearing targets, pretreatment of the NK cells with IL-2 leads to increased lysis of these targets and proximal signaling events appear to remain intact (7, 10). In contrast, little is known about the influence of IL-2 on NK cells that are fully inhibited in their killing of class I-bearing targets. Comparative analysis in this study showed clearly divergent influences of IL-2 on these two effector–target groups. Specifically, whereas IL-2 pretreatment of either NKL cells or cloned NK cells enhanced killing of partially resistant targets, IL-2 pretreatment did not alter the killing of fully resistant targets (Fig. 6*A* and data not shown). Interestingly, MHC class I expression on the target cells also had divergent effects on the signaling occurring in these two effector–target groups. Specifically, whereas inositol phosphate release (Fig. 6*B*) and elevations in $[Ca^{2+}]_i$ (data not shown) remained intact in NK cells encountering partially resistant targets, these PLC-γ-dependent signaling events were inhibited after NK cells bound fully resistant targets. Taken together, these results suggest there are fundamentally different signaling events in NK cells that are quantitatively versus qualitatively inhibited after class I recognition.

Table 1. Decreased killing after recognition of MHC class I$^+$ targets is not due to decreased conjugate formation

Effector cell	Target	Susceptibility to killing	Ab added	% NK cells in conjugate
NKL	C1R	+	None	15.5
		+	Anti-class I	16.7
		+	Anti-CD40	17.2
NKL	C1R-B27	−	None	12.1
		+	Anti-class I	12.9
		−	Anti-CD40	13.9
NK clone FZ3 (EB6$^+$)	C1R	−	None	27.6
	K562	+	None	26.9
NK clone FZ8 (EB6$^+$)	C1R	−	None	22.9
	K562	+	None	21.9

Sulfofluorescein diacetate-stained NK cell effectors were incubated with hydroethidine-stained targets. For Ab treatment, targets were preincubated for 10 min with F(ab')$_2$ fragments of either MHC class I-specific mAb (MB40.5) or CD40-specific mAb (G28-5). Conjugate formation was quantitated by two-color flow cytometry.

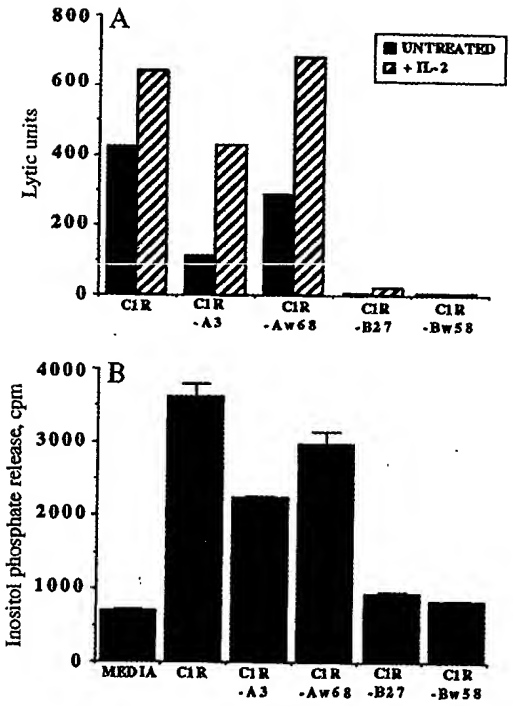

FIG. 6. Effects of IL-2 on EB6$^-$ NK cell signaling and cellular cytotoxicity. (*A*) Clonal EB6$^-$ NK cells were incubated for 18 hr in medium either without IL-2 or with 20 units of IL-2 per ml. These cells were then used as effectors against C1R cells or cells transfected with the gene for the indicated HLA molecules. (*B*) Inositol phosphate release was measured in *myo*-[^{3}H]inositol-labeled EB6$^-$ NK cells stimulated for 30 min with the indicated targets.

DISCUSSION

Studies that initially demonstrated an inverse correlation between the susceptibility of target cells to NK cell-mediated cytotoxicity and their level of class I expression lead to the proposal of two alternative models to explain the nature of the interaction between NK cells and MHC: the "target interference model" and the "effector inhibition model" (22). Subsequent findings have largely fulfilled the predictions made by the effector inhibition model (3). This model hypothesizes the presence of NK cell "inhibitory receptors" that specifically recognize class I molecules. Engagement of these receptors results in a "negative signal" that down-modulates NK cell-mediated killing of the class I$^+$ target. Indeed, several families of receptors have now been found on NK cells that specifically interact with certain class I molecules (2, 3, 25–27). Moreover, different subpopulations of NK cells within an individual vary in the family of MHC-recognizing receptors they express and in the MHC class I molecules they recognize (2, 3, 9). This differential expression creates a heterogeneous repertoire of NK cell reactivity against class I-bearing targets.

Despite the identification of MHC recognizing inhibitory receptors on NK cells, little is known about the mechanism(s) by which interaction between NK cells and class I$^+$ targets inhibits the development of the cytotoxic response. To evaluate this issue, we identified and characterized two experimental models in which class I recognition fully inhibited NK cell-mediated cytotoxicity. First, a recently isolated human NK

cell line, NKL, was unable to kill HLA-B27-transfected C1R cells. Similarly, GL183$^-$/EB6$^+$ NK cell lines were unable to lyse the HLA-Cw4$^+$ C1R target cells. For each of these effector populations, while susceptible targets elicited inositol phosphate hydrolysis and increased $[Ca^{2+}]_i$, the class I$^+$, NK-resistant targets did not trigger these signaling events. Blocking the interaction between MHC-recognizing receptors on the NK cells and MHC class I molecules on the targets, using anti-class I F(ab')$_2$ fragments, led to the restoration of the proximal PLC-γ-dependent signaling events and the subsequent generation of the cytotoxic response. These results strongly suggest that recognition of specific MHC class I molecules on target cells can potently inhibit the generation of pharmacologically active, inositol phosphate-derived second messengers critical to development of NK cell-mediated killing.

The mechanism by which MHC recognition inhibits the rapid activation of PLC-γ in NK cells remains unknown. Our demonstration that multiple proteins in NK cells are tyrosine phosphorylated after NK cell binding to class I$^+$ targets suggests that the inhibition is not global. However, this rapid PTK activation may or may not involve the specific proximal tyrosine kinases (i.e., lck, ZAP-70, and syk) that have been implicated in the tyrosine phosphorylation of PLC-γ in activated NK cells (28–31). Similarly, although MHC-recognizing receptors have been reported to be physically associated with certain subunits of trigger receptors (i.e., association of p58 molecules with ζ chains) (32), the functional consequence of these associations remains unclear. Theoretically, the sequestering of critical signaling elements could dramatically alter the normal activating response.

It should be emphasized that the inhibitory effects of MHC recognition on NK cell signal transduction were observed here in two systems where MHC conferred complete resistance to lysis. In contrast, partially resistant targets did not demonstrate reduced inositol phosphate release or calcium signaling (Fig. 6, ref. 10). Several factors could account for these differences. First, since clonal subpopulations of NK cells differ in the kinds of MHC-recognizing molecules expressed on their surface (3, 9), receptor-initiated signaling mechanisms are likely to differ between clones. In addition, depending on the NK clone, MHC recognition can be alternatively activating or inhibiting (8). Superimpose on this heterogeneity the fact that MHC-induced inhibition is qualitative in some cases and quantitative in others (9). The assay systems used here to measure signaling events may be sensitive to qualitative differences, but insufficiently sensitive to detect smaller quantitative changes. Specifically, the measurements of inositol phosphate release and elevated $[Ca^{2+}]_i$ used here are based on the average response of a whole population of cells. Interpretation of these kinds of assays is uncomplicated as long as there is a uniform response from each cell in the population. However, in the case of partial or intermediate responses by the cells, the generation of proximal signals in responding cells may mask signal inhibition in nonresponding cells. Identification of the MHC-recognizing receptors utilized in each of these experimental systems will help to differentiate between these alternative explanations.

Clearly, regulation of NK cell-mediated cytotoxicity is a multifactorial process. In addition to the inhibitory receptors, there are other "triggering receptors" and adhesion molecules that are important for NK cell activity (33). Furthermore, the absence of "triggering epitopes" on certain targets can make them resistant to NK cell-mediated killing (14, 17). Further studies are needed to determine not only the signaling pathways initiated by individual receptors but also how the multiple signals are integrated for regulation of NK cell responses.

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